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POSSIBLE MECHANISM FOR DENERVATION EFFECT ON WOUND HEALING

Annual Report

May 17, 1989

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Basic research is being conducted to investigate the role of transferrin, an iron-transport protein required for cell proliferation, in the neural effect on wound healing and tissue regeneration. The system of tissue repair under investigation is the regenerating limb of the axolotl, in which growth is strictly dependent on unknown factors from peripheral nerves. The rationale of the study is to measure and localize transferrin in normal and in denervated limb tissues, obtaining information with which to test the hypothesis that nerves contribute transferrin to cells of the regenerating tissues.

Before experiments of this nature can be undertaken, axolotl transferrin and antibodies against this factor must be available so that immunoassays can be developed to measure this protein in nerves, regenerating limbs, and other tissues from axolotls. During the first year of this project, transferrin was purified from axolotls and antisera against it were generated in mice and rabbits. Monoclonal antibodies were also prepared. During the second year (the period of this report), these antibodies have been used to develop an enzyme-linked immunosorbent assay (ELISA) for quantitative measurements of transferrin. Experiments have also begun using these antisera in immunocytochemical studies to localize transferrin in axolotl nervous tissue.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 85-23, Revised 1985).

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Table of Contents

Body of Report

Statement of the Problem under Study

The research supported by this contract relates to the influence exerted by peripheral nerves on cell proliferation and growth in innervated tissues. Although this influence has been recognized for many years, its biochemical basis is almost completely unknown. The medical importance of this problem lies in the frequent failure of wounds to heal at normal rates in tissues subjected to severe reduction in the nerve supply (1,2).

The animal model being used to study this problem is the urodele amphibian, which has a particularly well-developed capacity for tissue and organ regeneration (2). In this animal limbs are capable of complete regeneration in a process dependent on a neural influence for cell proliferation and growth of the new appendage (2,3). This developmental process is well-suited for investigations of the growth-promoting properties of nerves because the cellular events involved have been carefully characterized histologically (2) and because partial or complete denervation of the regenerating tissue is technically simple to accomplish, unlike various mammalian models of wound healing (3,4).

We are investigating the possible role of neural transferrin in the growth-promoting role of neurons. This factor, a glycoprotein used for iron-transport, is provided to cells via plasma and is required for cell proliferation, possibly because

of the iron cofactor requirement of enzymes controlling DNA synthesis (5,6). The recent finding that peripheral nerves are rich in transferrin (6,7), together with its importance for cell proliferation, suggest that an analysis of the availability and transport of this factor during vertebrate limb regeneration may provide useful new information on the role of nerves in the control of this process.

Background and Review of the Literature

Early studies, reviewed by Singer (3), indicated several basic features of the neural influence on limb regeneration: it can involve sensory, motor, or autonomic nerves, as well as central nervous tissue; it is needed for growth, but not for morphogenesis of the regenerating limb bud, called the blastema; it is mediated by release of protein factor(s) that are not species-specific. Recent attempts to identify the factor(s) in nervous tissue have used blastemas in organ culture to assay for factors in brain extracts capable of stimulating cell proliferation (8-11), but no protein has yet been purified from nervous tissue using this assay.

A similar *in vitro* assay has been used to investigate neural factors controlling growth and differentiation of embryonic myoblasts during muscle development (7, 12). Like limb regeneration, development of skeletal muscle is dependent on unknown protein factors released from nerve (13). Extracts of

chick brain or sciatic nerve, like co-culture with nerve explants, promote proliferation of cultured myoblasts and fusion of these cells into large, multinucleated myotubes which differentiate into muscle fibers (7). Using this bioassay, Oh and Markelonis (7) purified a protein from sciatic nerve capable of promoting the entire sequence of muscle differentiation in vitro. Upon characterization of this protein, which they designated "sciatin", it was found to be the iron-transport factor transferrin (7). Subsequent work showed transferrin to be abundant in peripheral nerves of birds (14) and mice (15). There is evidence that cultured neurons both take up transferrin by a receptor-mediated mechanism (16) and synthesize the protein (17). It is not yet known whether neural transferrin is critical for myogenesis in vivo and the physiological and developmental significance of transferrin in peripheral nerve remains to be established (6).

Using the cultured regeneration blastema bioassay, we showed in an earlier study that human serum transferrin is capable of stimulating the mitotic index, the DNA labeling index, and ^{3}H-thymidine incorporation (18). No effect was found with heat-denatured transferrin or with other serum proteins, such as albumin or immunoglobulin. The addition of transferrin at a concentration of 25 ug/ml to medium containing 1% fetal bovine serum caused all three parameters to increase to levels normally seen in medium containing 10% serum. The rate of blastema cell proliferation in medium with 10% serum is similar to that in vivo

and results in extensive outgrowth of cells from the explant. Concentrations of transferrin above the optimal dose were found to inhibit cell proliferation, an effect also observed in dose-response studies with mammalian cells (19). It has been suggested that this inhibitory effect at high transferrin concentrations is due to competition for receptors between iron-free transferrin and iron-carrying transferrin, resulting in reduced delivery of iron to the cells (20). In support of this hypothesis, we found that adding $FeCl_3$ to the medium relieved the effect of the high transferrin dose (18).

By means of an antiserum to Pleurodeles serum transferrin that cross-reacted with newt (Notophthalmus viridescens) transferrin, we demonstrated the presence of this protein in extracts of newt brain and peripheral nerve by immunodiffusion (21). Immunohistochemistry of newt ganglia and spinal cord indicated the presence of transferrin in neuronal perikarya and axons (Tomusk and Mescher, unpublished observations). That neural transferrin may stimulate the cell proliferation seen with brain extracts in the cultured blastema assay is indicated by the finding that removal of the iron from such an extract with the iron chelator desferrioxamine rendered the extract inactive, with full activity restored by the readdition of ferric iron (21). Moreover, the dose-response curve for brain extract was similar to that of transferrin and the inhibition of growth at high extract concentrations was reversed by $FeCl_3$ (21). This work suggests that transferrin is involved in the effect of brain

extracts on blastemal cell growth and prompted the present studies on the availability and delivery of transferrin during the proliferative phase of normal amphibian limb regeneration *in vivo*.

Rationale of the Study

The major technical objectives of this project are to purify transferrin from the urodele amphibian, the axolotl (*Ambystoma mexicanum*), to generate antibodies against this protein, and to develop an enzyme immunoassay for measuring concentrations of this protein in extracts of axolotl tissues. The rationale for this plan is that if significant release of transferrin from nerves occurs in the limb, then denervation of the limb should lower the transferrin concentration in limb tissues. Similarly, measurement of transferrin concentrations at different levels in ligated sciatic nerves should provide evidence regarding the possibility of axoplasmic transport of this factor.

It is also anticipated that the availability of homologous antiserum against axolotl transferrin will allow better immunohistochemical localization in nerves and regenerating limb tissues of this species than was possible with the antiserum against *Pleurodeles* transferrin used in our earlier studies.

Experimental Methods

(1) Development of an immunoassay for axolotl transferrin

The enzyme immunoassay developed for use in this project was of a different design from that proposed in the statement of work. It is more efficient and more sensitive than that proposed originally. Development of the modified immunoassay was prompted by two factors arising during the course of the project's first year. First, we had the opportunity to generate antiserum against axolotl transferrin in rabbits as well as in mice. The availability of antibodies from two species allowed greater flexibility in designing the immunoassay. Secondly, our department acquired an automated spectrophotometric reader for 96-well microtiter plates, which was interfaced with a microcomputer and printer. This allowed us to use 96-well plates rather than nitrocellulose in an enzyme-linked immunosorbent assay (ELISA). This made results available much more rapidly since they came directly from the plates.

ELISA's of various designs were tested for sensitivity and level of background. The best results were obtained with a noncompetitive, "sandwich"-type ELISA, similar to one described by Tijssen (22). Briefly, the steps involved in the assay procedure were as follows. Wells of a 96-well plate were coated with rabbit antiserum against axolotl transferrin at a dilution of 1:1500 overnight at 4° C. After this the plate was washed 3

times with Tris-buffered saline containing Tween (TBS/Tween). Wells were then blocked with 5% bovine serum albumin for 1 hour and washed again 3 times with TBS/Tween. Samples of supernatants from axolotl tissue homogenates were than added to the wells in triplicate along with standard concentrations of purified axolotl transferrin, also in triplicate. Plates containing samples and standards were incubated at 4° C overnight, after which they were washed again in TBS/Tween. Then mouse antiserum against axolotl transferrin was added at a dilution of 1:2000 and incubated at room temperature for 2-3 hours. Plates were then washed 3 times in TBS/Tween. A 1:500 dilution of secondary antibody (goat anti-mouse IgG), conjugated to alkaline phosphatase, was added to the wells and incubated 1 hour. Plates were washed 3 times with TBS/Tween. A substrate, para-nitrophenyl phosphate, was added at 1 mg/ml and incubated 30 min at room temperature. The color-generating reaction was stopped after 30 min by the addition of 3M NaOH and the plates were read at 405 nm and 540 nm with the dual wavelength plate reader (Model EL309, Bio-Tek Instruments). Protein content of the tissue extracts was determined by the bicinchoninic acid method of Smith et al. (23).

This rabbit antibody/transferrin/mouse antibody sandwich technique with adsorption to 96-well plates offers a high level of sensitivity and low background with very simple and fast quantification of the bound transferrin. Appropriate software (Bio-Tek Instruments) was used to log the data on a floppy disk directly from the plate reader and to generate standard curves by

linear regression. A typical standard curve from one experiment is shown in Figure 1.

(2) Immunocytochemistry and electron microscopy of axolotl nerves

Late during the period of this report, we began light microscopic studies using mouse antiserum against axolotl transferrin to localize this protein in axolotl peripheral nerve by indirect immunocytochemistry. Three adult axolotls were anesthetized in 2% benzocaine solution and perfused via the heart with 4% paraformaldehyde. Brains, brachial regions of the spinal cord, and dorsal root ganglia with attached brachial nerves were removed, along with liver as a positive control for transferrin staining. The central nervous system tissues, 5 nerves and ganglia, and samples of liver were placed in OCT compound and rapidly frozen at -40° C in dry ice/isopropanol. These were sectioned to 20 micron thick sections on a cryostat. These sections were incubated overnight with a 1:100 dilution of antiserum, washed in TBS and stained indirectly with a secondary antibody conjugated to biotin and avidin conjugated to alkaline phosphatase, using the ABC Vectastain kit from Vector Laboratories. Controls employed have included preimmune serum, no serum, and no secondary antibody. We have begun to obtain good results with this method, which has the advantage of allowing simple counterstains and observation with a bright-field microscope rather than a fluorescence microscope, which we also

used in preliminary work earlier this year. Background from autofluoresence is eliminated and, unlike fluorescence, staining is permanent. It is expected that the avidin-biotin conjugate Vectastain method, rather than immunofluorescence, will continue to be the principal method used for immunolocalization during the rest of the project.

Other nerves and ganglia were embedded in Araldite, thin sectioned and processed for observation by transmission electron microscopy by routine methods.

Results

(1) Immunoassay of transferrin

The transferrin content of larval axolotl forelimb regeneration blastemas was measured by the ELISA method described above. As indicated in Table I, the concentration of this protein in distal regions of mid-bud stage blastemas (6 days after amputation) was not significantly different from that in complete, unamputated limbs. Surgical devascularization of limbs or mid-bud stage blastemas by transection of the brachial artery and vein had no consistent significant effect on transferrin concentration one day later (data not shown), probably due to the fact that circulation was re-established in the limb by this time. However, transection of the brachial nerves near the brachial plexus one day before the blastemas were sampled (5 days postamputation) reduced the transferrin concentration in distal tissues by approximately 50% (Table I, Figure 2). This effect of

denervation was similar if nerves were transected as early as 3 days postamputation or even on the day of amputation. These observations indicate that the adverse effect of denervation on the transferrin concentration in the limb is rapid and lasts at least 6 days. The latter result is consistent with our earlier finding that denervated and amputated larval forelimbs are not reinnervated distally for 10 days (24).

Denervation of larval Ambystoma forelimbs at any early stage of regeneration leads to a rapid decrease in cell cycling and growth. It is also known that transferrin uptake is greatly increased as cells begin to proliferate. It is possible therefore that the reduced concentration of transferrin after denervation is related to reduced proliferative activity rather than to a reduction in the content of nerves. To test this possibility, larvae were X-irradiated (2000 rads) with one forelimb shielded and forelimbs were amputated bilaterally one day later. As expected, the X-irradiated limb failed to regenerate, while the shielded limb underwent blastema formation normally. Six days later, when the control limbs were at the mid-bud stage of regeneration, distal regions of both forelimbs were sampled. As shown in Figure 3, inhibition of growth by X-rays had no effect on transferrin content. This control experiment strongly suggests that the 50% decrease in the transferrin content of denervated limb stumps is due to the loss of axons from the tissue, which is consistent with our hypothesis that nerves are an important source of transferrin for blastemal cells.

Measurements of the transferrin concentration in tissues of adult axolotls indicate that peripheral nerve is relatively rich in this factor compared to most tissues, which has also been shown for mice (15). In preliminary experiments, we have begun to test the effect of ligation on the content of transferrin in 2 mm segments along the sciatic nerve. Results from one such experiment are shown in Table II. The mean transferrin concentration in four segments proximal to the ligature was 210% of that in control segments 1 cm from the ligature. The segment distal to the ligature contained 179% of the control concentration. These preliminary results suggest that transferrin is transported axonally in both the anterograde and retrograde directions. We expect additional experiments currently underway will confirm this result. Such a finding would support the hypothesis regarding a role for transferrin in the trophic effect of peripheral nerves. Since we are measuring a plasma protein, it is important to investigate whether edema at a ligature is contributing transferrin to the accumulation there. Control experiments for the axonal transport study will therefore include the use of inhibitors of axonal transport to determine the involvement of edema in the accumulation.

(2) Localization of transferrin in peripheral nerves

As soon as rabbit antiserum to axolotl transferrin was available, in the latter part of the first year of this project, it was used with a fluorescein-conjugated secondary antibody in

attempts to localize transferrin in teased whole-mount preparations of brachial nerve. The results showed positive staining for transferrin in both axons and the sheath of Schwann cells surrounding axons (Figure 4). Nerves stained with a similar dilution of control (pre-immune) rabbit serum were completely negative (not shown). These immunocytochemical results are very similar to those obtained by others with chick peripheral nerve (25).

Recently we have begun to examine transferrin in sectioned axolotl brachial nerves, rather than teased whole-mounts, so that the connective tissue and vascular components could be included. As indicated in the Methods section, mouse antiserum and a biotinylated secondary antibody procedure is being used for these studies. Although micrographs were not yet available for this report, the results confirm the presence of transferrin in axons and Schwann cells. Blood vessels also stain for transferrin, as expected. Like other connective tissues, epineurium and perineurium stain positively for transferrin. Staining is more intense in these connective tissues of nerve than in the endoneurium.

It is well-established that the perineurium is a major component of the blood-tissue barrier which regulates availability of plasma proteins and other factors to the endoneurium, Schwann cells and axons. The barrier consists of flattened cell processes of the perineurial fibroblasts which are joined by tight junctions (26). Transport across the flattened

cell processes is apparently due to receptor-mediated endocytosis and release on the other side. The localization of transferrin in the perineurial processes at the light microscope level may indicate that this tissue is involved in uptake of transferrin from interstitial fluid outside the nerve and delivery of this factor to the endoneurial environment, an idea we would like to test. Routine transmission EM examination of the perineurium shows the processes to contain numerous micropinocytotic vesicles (Figure 5), indicating extensive uptake and transport of material in these cells.

Discussion and Conclusions

The primary reagents generated in the first phase of this project, the antibodies against axolotl transferrin, have been used successfully during the second phase to develop a rapid and sensitive ELISA for measurement of transferrin in tissue extracts and to localize this protein in tissue sections for light microscopy. Both the immunoassay method and the immunocytological technique are of slightly different designs than those originally proposed in the statement of work, but the changes made have resulted in several significant advantages that were not apparent when the statement of work was prepared.

The ELISA has been used to show that denervated limb stumps contain approximately half as much transferrin as regenerating limbs or intact limbs. Control experiments involving the use of

X-irradiation rather than denervation to inhibit regenerative growth indicate that the reduced transferrin concentration is due to loss of nerves and not to the lack of proliferating cells. Temporary devascularization of limb stumps had no significant effect on transferrin content of the limbs one day after the operation. This result may be due to the extremely rapid revascularization of these limbs, which precludes direct comparison with the denervation experiments.

The same assay has also been used to measure transferrin content in ligated sciatic nerves of adult axolotls. These preliminary experiments suggest that this protein is transported within axons in both directions, a result that would be consistent with other data in the literature. Together, these findings from the ELISA support the hypothesis that axons deliver a significant amount of transferrin distally in axolotl limbs. Further ligated sciatic nerve studies <u>in vivo</u>, currently underway, will include more sophisticated experiments than those done to date and will clearly demonstrate the extent and velocity of axonal transport of transferrin in this model system.

Immunocytochemical work has extended the preliminary studies done one year ago and has implicated the perineurium in the uptake of transferrin by peripheral nerve. Involvement of this nerve-tissue barrier in physiology of neural transferrin has not been considered previously in this field, but is consistent with what is known regarding uptake of plasma proteins by peripheral nerve. Additional light microscope immunocytochemistry

planned for the third year of the project, and control experiments with other neuronal proteins, will clarify exactly which cells of nerve contain transferrin.

It should be noted that nothing has been published by other laboratories in the past year that would weaken the hypothesis being tested by this project: that the trophic effect of peripheral nerves involves release of transferrin. On the contrary, our early studies on the stimulatory effect of this factor on cultured blastema cells have been confirmed by a group in France (27).

In conclusion, the project is on schedule and refinements made in the ELISA and in the staining techniques developed this year will not only allow the remaining technical objectives to be achieved more rapidly and efficiently, but will also allow more sophisticated questions involved in these objectives to be addressed.

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Table I

Transferrin Content of Larval Axolotl Forelimbs

Tissue	ng Transferrin / ug Protein (mean ± S.E.)
Unamputated Limbs (n=6)	7.48 ± 1.31
Mid-Bud Regenerate (6 Day) (n=8)	8.98 ± 3.03
6 Days Postamp., Denervated Day 5 (n=5)	4.15 ± 2.24
6 Days Postamp., Denervated Day 3 (n=5)	2.95 ± 1.07
6 Days Postamp., Denervated Day 0 (n=8)	4.25 ± 1.69

Table II

Transferrin Content of Axolotl Sciatic Nerve

region of nerve	ng transferrin / ug protein (mean ± S.E.)	% of Control
control (1 cm from lig.)	10.94 ± 1.76 (n=6)	100
prox to ligature	22.98 ± 3.33 (n=4)	210
distal to ligature	19.61	179

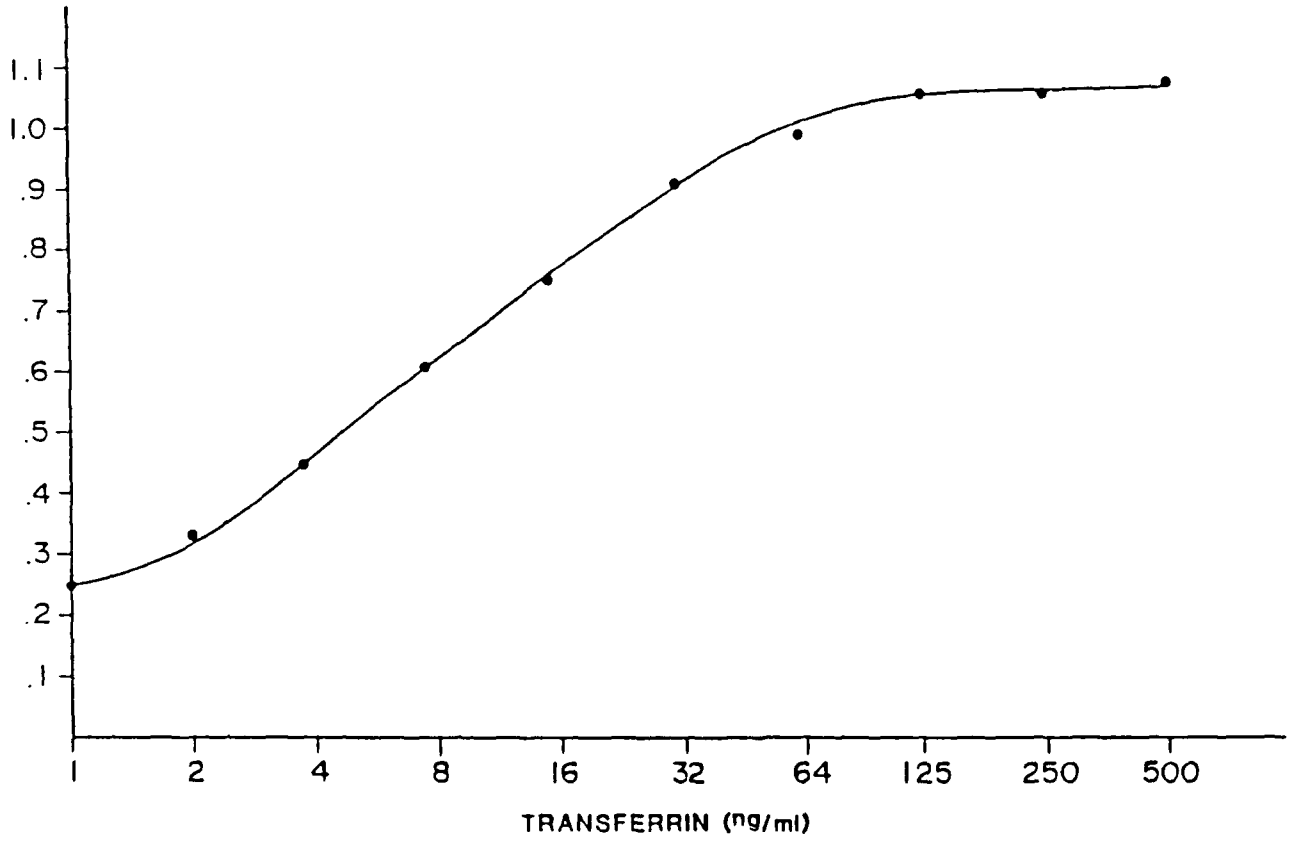

Figure 1. Standard curve of enzyme-linked immunosorbent assay (ELISA) used to quantitate axolotl transferrin.

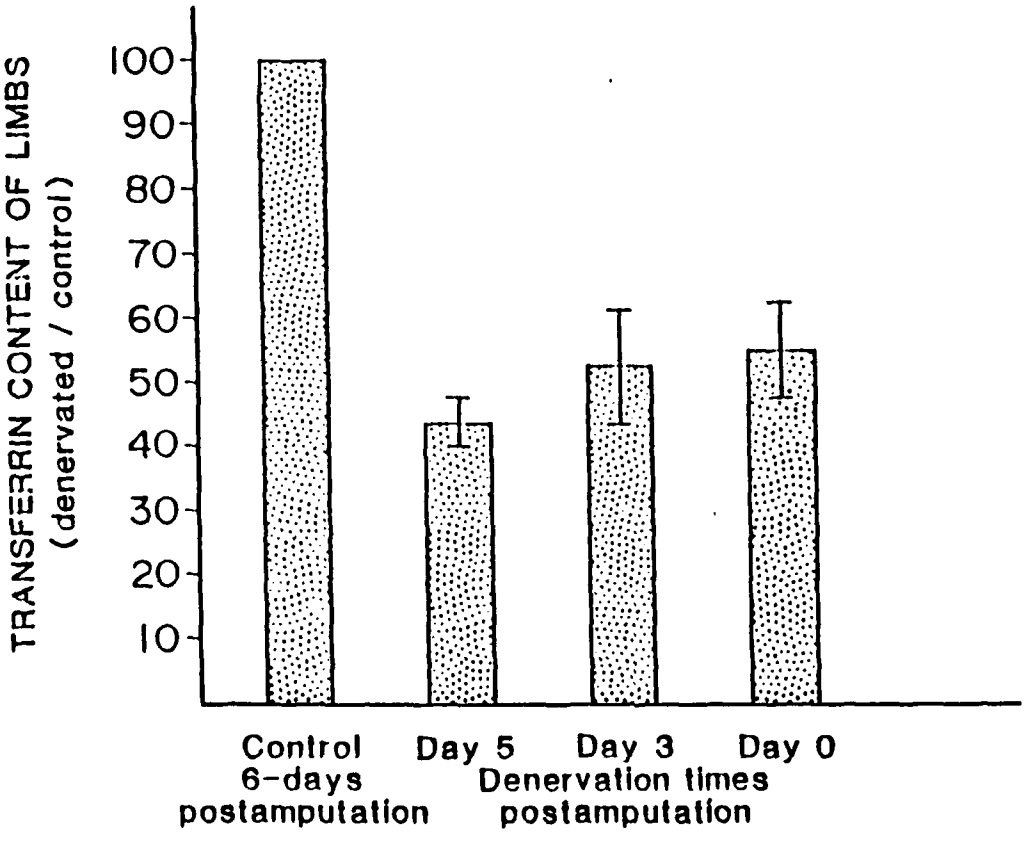

Figure 2. Transferrin content in distal regions of mid-bud stage blastemas and contralateral denervated, nonregenerating stumps of larval axolotl forelimbs.

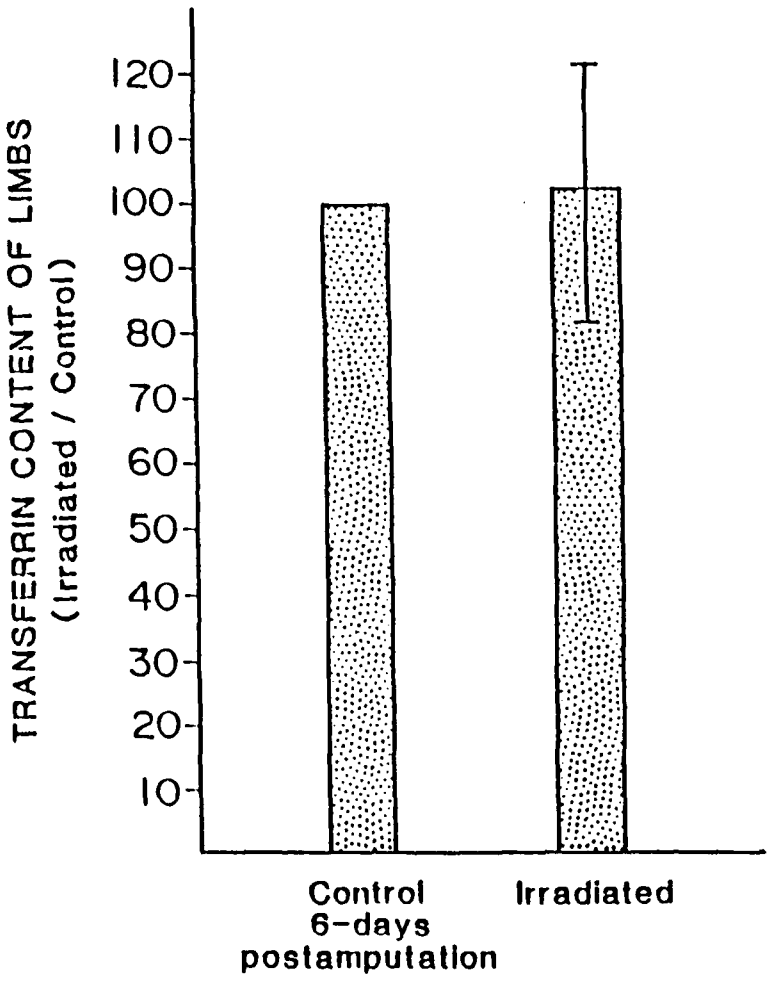

Figure 3. Transferrin content in distal regions of mid-bud stage blastemas and contralateral x-irradiated, nonregenerating stumps of larval axolotl forelimbs.

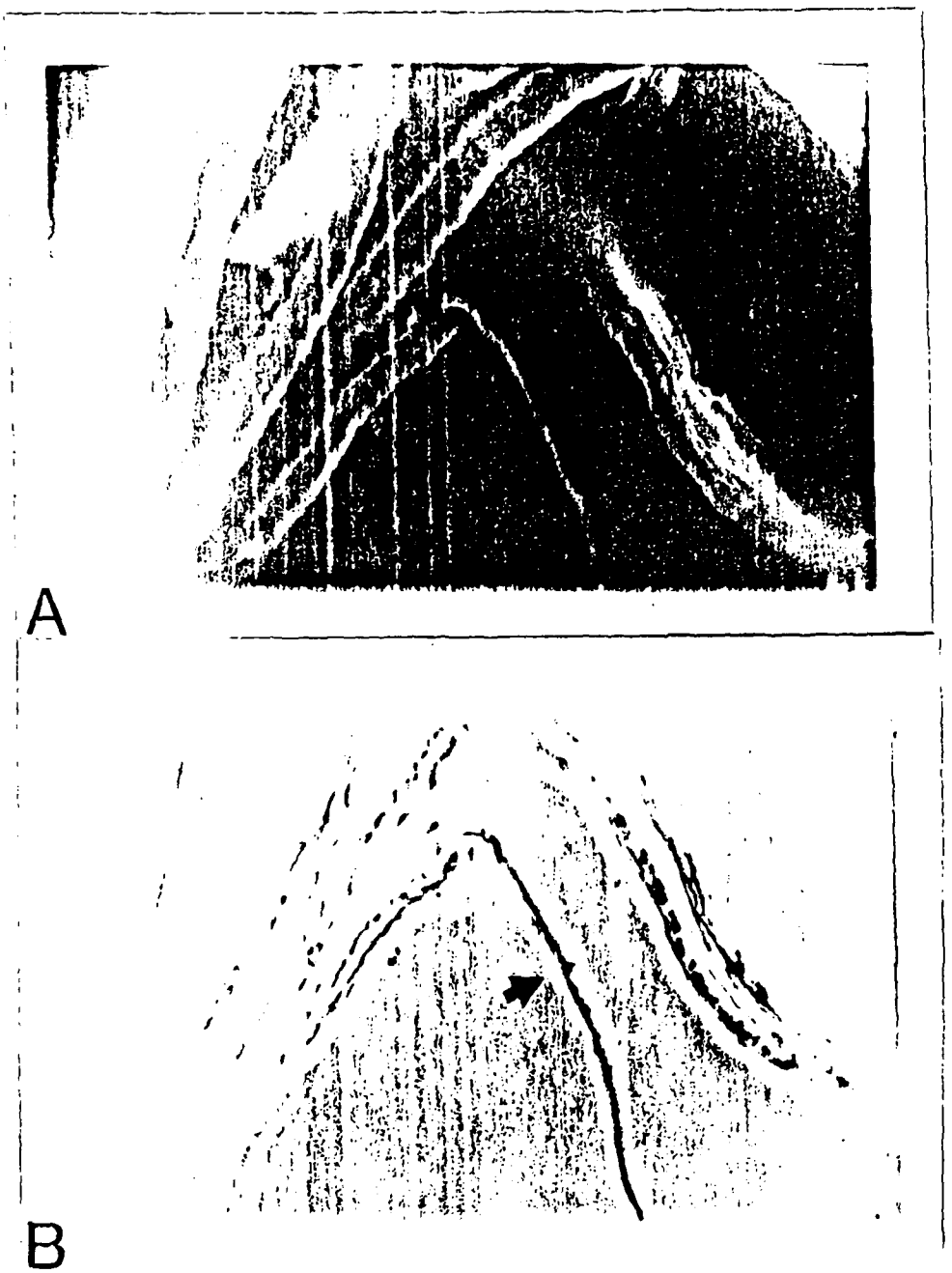

Figure 4. (A): Indirect immunofluorescence of transferrin in Schwann sheath and axons of a teased brachial nerve preparation from axolotl. (B): Phase-contrast image of same field, with arrow indicating an axon. (75X)

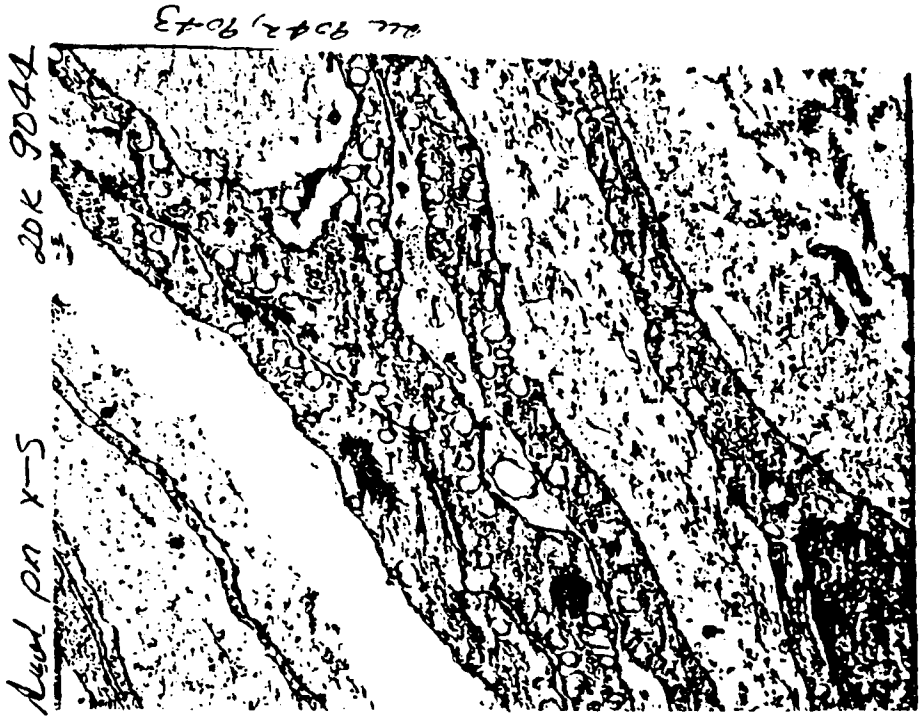

Figure 5. Electron micrograph of an axolotl brachial nerve perineurium showing four flattened fibroblastic processes with numerous micropinocytotic vesicles. (20,000X)